Letter

Continued antigenic variation of highly pathogenic avian influenza A (H7N9) virus in laying hens in China, 2020–2021

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Dear Editor,

Since it first appeared in humans in China in 2013, a novel H7N9 influenza virus has caused 1,568 cases of infection, with a mortality rate of 39.2% (Yin et al., 2021). The early H7N9 virus strains had low pathogenicity and did not cause avian disease (Zhang et al., 2013). However, they caused concern about poultry consumption and caused huge economic losses to the poultry industry. Since mid-2016, some H7N9 viruses mutated, with an insertion of four basic amino acids in their cleavage sites of hemagglutinin (HA) and thereby became highly pathogenic to chickens, causing multiple outbreaks in poultry in China (Wang et al., 2017; Hou et al., 2019). The H7-Re1 vaccine, which contains antigens against the HA and neuraminidase (NA) produced by A/pigeon/-Shanghai/S1069/2013, was released by the Chinese government in September 2017, successfully controlling the transmission of H7N9 viruses in both human beings and poultry (Shi et al., 2018). We reported in 2019 that the H7-Re2 vaccine, which was derived from A/chicken/Guangdong/SD098/2017 and initially used in December 2018, was poorly protective against novel H7N9 variants with antigenic drift (Jiang et al., 2020). By accumulating amino acid changes, the antigenicity of HA protein was changed, and this antigenic drift allowed influenza viruses to escape recognition by neutralizing antibodies, which had a direct impact on vaccine effectiveness. In June 2020, the H7-Re2 vaccine was replaced by H7-Re3, a recombinant bearing the *HA* and *NA* genes of A/chicken/Inner Mongolia/SD010/2019 and internal gene segments of *PR8*.

During active surveillance of avian influenza virus infections in China in 2020 and 2021, 13 strains of H7N9 viruses were identified in 11,520 swab samples collected from laying hens in 14 provinces. Strains were isolated by inoculation into 9–11-day-old specific pathogen-free (SPF) chicken embryos and identified by reverse transcription polymerase chain reaction (RT-PCR) and sequencing. The characteristics of the 13 virus strains are shown in Supplementary Table S1. The strains were isolated from Hebei, Shanxi, and Yunnan provinces. The 2019 H7N9 variants first arose in Hebei Province, and those provinces have become the main foci of the H7N9 virus.

Sequencing of the complete genomes of the H7N9 strains has been previously described (Hoffmann et al., 2001) and deposited in GISAID database under accession no. EPI1986929–EPI1986935 and EPI1987114–EPI1987210. In all 2020 and 2021 strains, HA includes multiple basic amino acids (PKRKRIAR/GLF or PKRKRTAR/GLF) at the cleavage site, suggesting that they are associated with high pathogenicity in chickens. We have not detected the low-pathogenicity H7N9 viruses since 2019, nor have other laboratories. They may have disappeared and been replaced by highly pathogenic viruses. We selected three strains for the evaluation of the pathogenicity in SPF chickens and ducks that were H7N9 antibody-negative. The strains had intravenous pathogenicity index values of 3.00 in chickens, confirming their high pathogenicity. On the other hand, they had low pathogenicity in ducks, in which no clinical signs or symptoms were observed during the 14-day observation period. Furthermore, virus replication in the lungs, intestine, liver, spleen, kidneys, and brain of inoculated ducks was very limited, and few ducks shed virus on day 3 (Supplementary Table S2). The result was consistent with those of Yin et al. who reported that the H7N9 viruses isolated after 2019 had low pathogenicity in ducks (Yin et al., 2021). The limited replication in waterfowl has contributed to virus control and elimination.

The receptor binding sites in the HA protein of 13 strains were identified as T160, V186, Q226, and G228 (H3 numbering, except E228 for HD1229), suggesting that all 13 strains could bind avian and human receptors (Zhang et al., 2013). Yin et al. found that, compared with low-pathogenicity H7N9 viruses, highly pathogenic strains bound primarily to avian receptors and had reduced affinity for human receptors (Yin et al., 2021). In addition to the reduced prevalence of the virus, that may be the reason for the decreased number of human cases caused by H7N9 virus.

Phylogenetic analysis based on the *HA* gene (Fig. 1) shows that all the viruses belong to a high-pathogenicity H7N9 clade in which the strains isolated in 2019 are clustered. Previous studies reported that the H7N9 high-pathogenicity AIVs in China comprised ten genotypes (Yin et al., 2021). The genomic similarity to strains previously isolated in other

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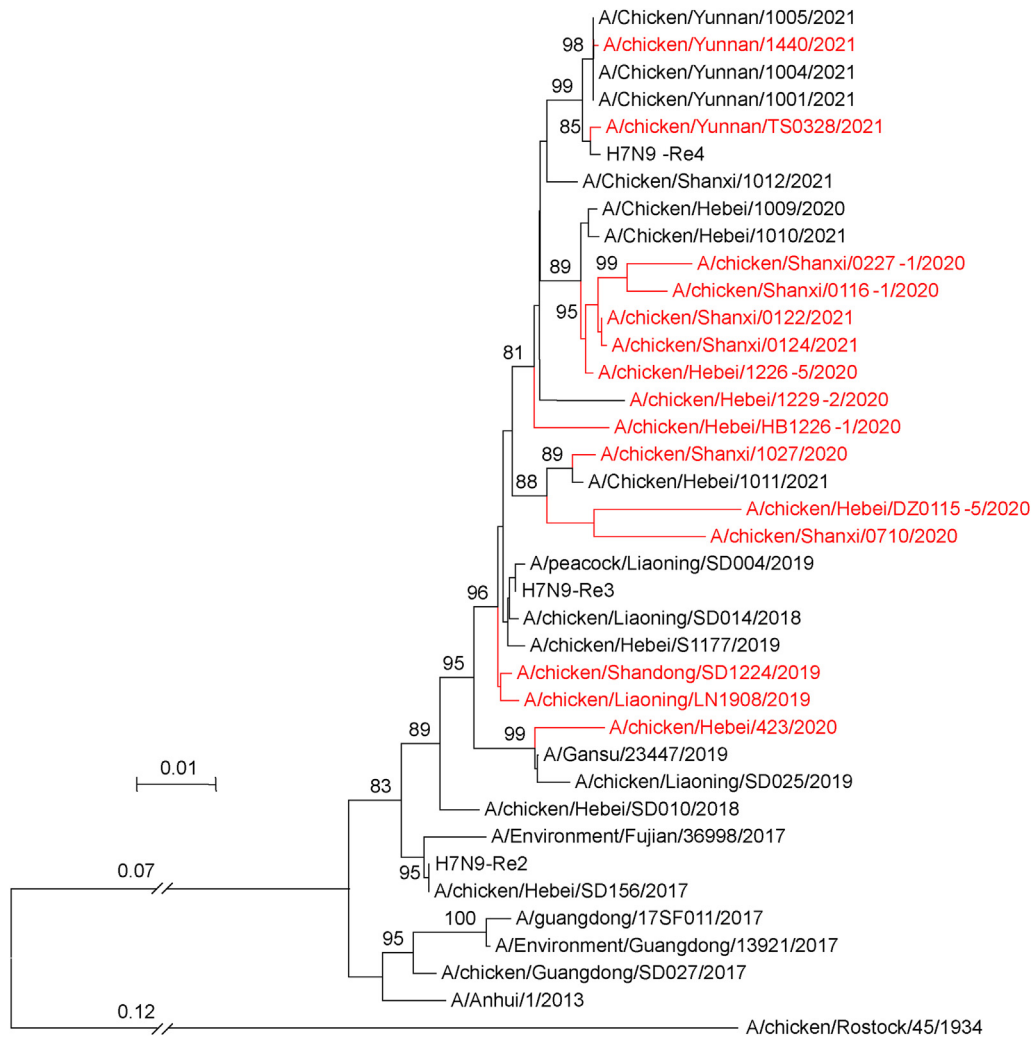
Fig. 1. Phylogenetic analyses of the *HA* genes of H7N9 highly pathogenic avian influenza viruses. Trees were constructed with MEGA5.10 software using the neighbor-joining method. Bootstrap analysis was performed with 1000 replications. The viruses sequenced in this study are shown in red in the phylogenetic trees. Scale bars indicate nucleotide substitutions per site.

laboratories indicates that the 13 H7N9 high-pathogenicity AIVs isolated in this study can be placed into two genotypes. Twelve of the 13 strains were classified as genotype 2 that was previously reported by Shi et al. (2018) and Yin et al. (2021). TS0328 is a novel genotype, most likely a reassortant virus resulting from genotype 2 and H9N2 viruses, which we assigned to genotype 11 (G11, Supplementary Table S3). The *PB2*, *PB1*, *PA*, *NP*, and *NS* of TS0328 and the other 12 viruses are located in different groups in the phylogenetic trees (Supplementayr Fig. S1–S7). Genotype 2 is the current prevailing genotype. The *HA* genes of the 13 H7N9 viruses share 95.6%–98.0% identity to each other and 94.9%–99.6% identity with H7-Re3 at the amino acid level.

To investigate the antigenicity of the virus strains and the current H7-Re3 vaccine, antisera were prepared by immunization of SPF chickens with inactivated allantoic fluid containing the respective virus. The 12 viruses isolated in 2020–2021 cross-reacted poorly with antiserum against H7-Re3, and the hemagglutination inhibition (HI) antibody titers were 8–32-fold lower than those against H7-Re3 antigen. Only the HeB423 virus cross-reacted well with antiserum against H7-Re3, and its HI antibody titer was 4-fold lower than those against H7-Re3 (Supplementary Table S4). The HI data were further analyzed quantitatively by antigenic cartography (Smith et al., 2004), and the viruses formed three different antigenic groups. The first (antigenic group I) contained the vaccine seed virus H7-Re3 and six viruses isolated in 2020 and 2021. The second (antigenic group II) contained six viruses isolated in 2020 and

2021. The third (antigenic group III) contained only one virus isolated in 2020 (Supplementary Fig. S8). The results indicated that the H7N9 strains isolated in 2020–2021 exhibited rapid antigenic drift and had obviously different antigenicity from the H7-Re3 strain. Previous studies showed that the *N*-linked glycosylation sites at positions 133–135 and 158–160 on HA resulted in the antigenic drift of H7N9 viruses (Yin et al., 2021). However, the differences in the numbers of glycosylation sites between 13 isolates and H7-Re3 were not significant; DZ0115-5 and SX0227-1 lacked a glycosylation site at position 133–135. There may be reasons for the antigenic drift other than differences in glycosylation sites. The results of cross-reactive HI studies also showed that HY1027 virus antisera had a broader cross-reactivity to other strains. Therefore, we generated a recombinant rHY1027 virus, bearing the *HA* and *NA* genes of HY1027 strain with six internal genes of PR8, as a vaccine candidate.

To verify the protective efficacy of the two vaccines, SPF chickens were immunized with the H7-Re3 vaccine and rHY1027 vaccine. HY1027, Y1440, and SX0227-1 were selected for challenge studies. During a 10-day observation period, chickens challenged with HY1027, Y1440, and SX0227-1 all died within three days. H7-Re3-vaccinated chickens had clinical symptoms, such as high fever, depression, and decreased feed and water intake. Moreover, 80% of the chickens shed viruses in tracheal and cloacal swabs on days 3 and 5 post-challenge, and 20.0%–40.0% of the challenged birds died (Table 1). The poor protection

Table 1
Protective efficacy of H7-Re3 and rHY1027 vaccines against H7N9 viruses in chickens[a].

Group	Vaccine	HI titer ± SD, log$_2$		Challenge test results, by swab type, no. positive birds/no. tested (mean titer ± SD)[b]				No.surviving birds/no. total
				3 dpi		5 dpi		
		H7-Re3	Challenge virus	Tracheal	Cloacal	Tracheal	Cloacal	
HY1027	H7-Re3	9.1 ± 0.36	5.6 ± 0.32	2/10 (2.63 ± 0.56)	7/10 (3.17 ± 0.76)	5/10 (2.83 ± 0.62)	8/10 (4.63 ± 0.97)	8/10
	rHY1027	8.0 ± 0.33	9.3 ± 0.35	0/10	0/10	0/10	0/10	10/10
	Mock	<1.0	<1.0	5/5 (5.76 ± 1.25)	5/5 (6.38 ± 1.33)	ND	ND	0/5
Y1440	H7-Re3	9.2 ± 0.39	4.3 ± 0.30	4/10 (2.93 ± 0.63)	8/10 (3.46 ± 0.83)	6/8 (3.12 ± 0.83)	8/8 (4.87 ± 1.12)	6/10
	rHY1027	7.8 ± 0.36	7.5 ± 0.34	0/10	0/10	0/10	0/10	10/10
	Mock	<1.0	<1.0	5/5 (5.73 ± 1.23)	5/5 (6.68 ± 1.46)	ND	ND	0/5
SX0227-1	H7-Re3	9.1 ± 0.40	5.9 ± 0.34	3/10 (2.58 ± 0.49)	7/10 (3.13 ± 0.67)	5/10 (2.78 ± 0.59)	8/10 (4.65 ± 0.87)	8/10
	rHY1027	7.8 ± 0.33	8.2 ± 0.35	0/10	0/10	0/10	0/10	10/10
	Mock	<1.0	<1.0	5/5 (5.53 ± 1.12)	5/5 (6.43 ± 1.67)	ND	ND	0/10

HI, hemagglutination inhibition assay; *dpi*, days post-infection; *ND*, not done; *SD*, standard deviation.
[a] Chickens were immunized with the H7-Re3 or rHY1027 vaccine, and HI antibody titers were determined on day 21 post-vaccination.
[b] Chickens were challenged with 10^6 50% egg infectious dose (EID$_{50}$) of each virus; virus titers are expressed as log$_{10}$ EID$_{50}$/0.1 mL.

of the H7-Re3 vaccine against HY1027, Y1440, and SX0227-1 indicated that the H7-Re3 vaccine was unable to prevent infection by the currently circulating H7N9 viruses.

On the contrary, all rHY1027-vaccinated chickens survived without any clinical signs and symptoms or detectible virus shedding in tracheal or cloacal swabs on days 3 and 5 after challenge (Table 1). The rHY1027 vaccine candidate had good protection against homologous and heterologous viruses.

Vaccination successfully controlled the infection of both poultry and humans by the H7N9 virus in China and resulted in a sharp decline in the prevalence of the virus. However, mass vaccination poses a series of questions, such as accelerated variation of antigens (Wu et al., 2021). For example, the vaccine against H7N9 has been updated twice in three years, and since January 2022, H7-Re3 vaccine was replaced with H7 Re4 vaccine. In addition to developing new antigen-matched vaccines, we should implement measures to eliminate highly pathogenic H7N9 viruses.

Footnotes

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2022.04.012

References

Hoffmann, E., Stech, J., Guan, Y., Webster, R.G., Perez, D.R., 2001. Universal primer set for the full-length amplification of all influenza a viruses. Arch. Virol. 146, 2275–2289.
Hou, G., Li, J., Wang, S., Cheng, S., Peng, C., Chen, J., Jiang, W., 2019. Hemagglutinin characteristics, changes in pathogenicity, and antigenic variation of highly pathogenic h7n9 avian influenza viruses in China. J. Infect. 78, 158–169.
Jiang, W., Hou, G., Li, J., Peng, C., Wang, S., Liu, S., Zhuang, Q., Yuan, L., Yu, X., Li, Y., Wang, J., Liu, H., 2020. Antigenic variant of highly pathogenic avian influenza a (H7N9) virus, China, 2019. Emerg. Infect. Dis. 26, 379–380.
Shi, J., Deng, G., Ma, S., Zeng, X., Yin, X., Li, M., Zhang, B., Cui, P., Chen, Y., Yang, H., Wan, X., Liu, L., Chen, P., Jiang, Y., Guan, Y., Liu, J., Gu, W., Han, S., Song, Y., Liang, L., Qu, Z., Hou, Y., Wang, X., Bao, H., Tian, G., Li, Y., Jiang, L., Li, C., Chen, H., 2018. Rapid evolution of h7n9 highly pathogenic viruses that emerged in China in 2017. Cell Host Microbe 24, 558–568 e557.
Smith, D.J., Lapedes, A.S., de Jong, J.C., Bestebroer, T.M., Rimmelzwaan, G.F., Osterhaus, A.D., Fouchier, R.A., 2004. Mapping the antigenic and genetic evolution of influenza virus. Science 305, 371–376.
Wang, N., Sun, M., Wang, W., Ouyang, G., Chen, Z., Zhang, Y., Zhao, B., Wu, S., Huang, J., Sun, H., Liao, M., Jiao, P., 2017. Avian influenza (h7n9) viruses co-circulating among chickens, southern China. Emerg. Infect. Dis. 23, 2100–2102.
Wu, Y., Hu, J., Jin, X., Li, X., Wang, J., Zhang, M., Chen, J., Xie, S., Qi, W., Liao, M., Jia, W., 2021. Accelerated evolution of h7n9 subtype influenza virus under vaccination pressure. Virol. Sin. 36, 1124–1132.
Yin, X., Deng, G., Zeng, X., Cui, P., Hou, Y., Liu, Y., Fang, J., Pan, S., Wang, D., Chen, X., Zhang, Y., Wang, X., Tian, G., Li, Y., Chen, Y., Liu, L., Suzuki, Y., Guan, Y., Li, C., Shi, J., Chen, H., 2021. Genetic and biological properties of h7n9 avian influenza viruses detected after application of the h7n9 poultry vaccine in China. PLoS Pathog. 17, e1009561.
Zhang, Q., Shi, J., Deng, G., Guo, J., Zeng, X., He, X., Kong, H., Gu, C., Li, X., Liu, J., Wang, G., Chen, Y., Liu, L., Liang, L., Li, Y., Fan, J., Wang, J., Li, W., Guan, L., Li, Q., Yang, H., Chen, P., Jiang, L., Guan, Y., Xin, X., Jiang, Y., Tian, G., Wang, X., Qiao, C., Li, C., Bu, Z., Chen, H., 2013. H7n9 influenza viruses are transmissible in ferrets by respiratory droplet. Science 341, 410–414.